Synopsis

Medicinal plants, their preservation, identification, synthesis, quantification and quality control of natural products have potentiated present day herbal drug, pharmaceuticals and neutraceutical industries in an unprecedented manner. The present study is centered around some selected high-value plants, *(Hemidesmus indicus*, *Tylophora indica, Cryptolepis buchananii*) of the Apocynaceae family with both traditional acceptance and commercial presence. Considering their pharmaco-therapeutic importance plus overexploitation scenario the present work has recommended fast and effective micropropagation technique, using MS media-PGR combination, which is clubbed with *in-planta* elicitation to check its role in biomass generation and marker compound accumulation. As root is the main part for marker accumulation in all these three plants impact of different variables on root length has been analyzed. MS- BAP (1.5 mg/L) combo has produced the highest (6.7) number of shoots/ explant and average shoot length was 11.7 cm in case of *H. indicus* which are 4.1 and 9.2cm respectively for *T. indica*. In *C. buchananii,* Kin (1mg/L) generated maximum shoot length (6.8 cm) despite of a slow growth trend. In all plants IBA 1.5 mg/L have promoted rooting phenomenon more than the other combinations of Ms+ auxin. Profuse base callus formation and increase in fresh weight of NMs has been noted in *T. indica* which may be connected with lower multiplication rate. Methyl jasmonate (50 µM) based elicitation of healthy microshoots has yielded promising result in *T. indica* and *H. indicus* but both these phenomenon were absent in *C. buchananii.* ISSR marker based genetic fidelity assessment has been done in support of the monomorphic plant production trend in tissue culture. Phytochemical screening has revealed the TPC, TFC, TTC, PPC content in mother plants and when the values were compared with the *in-vitro* ones significant content wise compatibility between the two has been observed in these plants except *C. buchananii*. The activity of antioxidant enzymes (SOD, CAT, APX), DPPH, FRAP assays plus pancreatic lipase inhibitory activities has confirmed the assumption that *in-vitro* raised plants can be a sustainable option against the wild plants in terms of basic bioactivity and phytochemical content. This idea was further supported by the rapid, validated, and sensitive densitometric HPTLC based quantification of marker compounds in these plants where elicitor treated root culture exhibited higher content of the marker. For *H. indicus* and *T. indica* the values of these experiments were noteworthy but for *C. buchananii* naturally grown samples have shown better result when tested for various attributes. In case of anti-bacterial activity only marker compounds has shown significant result. The present work is more of a multifaceted screening which has revealed many aspects of these medicinal plants for the very first time and highlighted prospective application oriented role of these medicinal plants.

Introduction

The identification, synthesis, commercially acceptable validation and optimization of the plant derived natural products are the key ingredients of pharmaceuticals, nutraceuticals, cosmetics cum herbal product based industries, which are experiencing a rapid expansion in recent days (Faustino et al., 2019). Secondary metabolites (SMs), of various chemical categories, control the application or bioactivity of commercially valuable medicinal and aromatic plants (MAPs) (Mendoza and Silva., 2018). So, the techniques or experimentation aiding in enhancement in natural product synthesis or accumulation of higher biomass has its own commercial importance (Velu et al., 2018). Interestingly, plant tissue culture has emerged as a perpetual strategy for production cum standardization of industrially important bioactive compounds (Chandran et al., 2020). Moreover, it is important as a conservation tool for endangered flora (Hussain et al., 2012). An expansive review by Anand et al., 2020 has elaborately discussed the broad-spectrum role of medicinal plants of the one of the major plant family, Apocynaceae. In the present study, some of the medicinal plants i.e. *Hemidesmus indicus* L. Br. (*Anantamul* or Indian Sarsaparilla), *Tylophora indica* (Burm. f.) Merrill (*Antamul* or Indian ipecac) and *Cryptolepis buchanani* Roemer et Schultes (*Krishna sariva* or Wax leaved climber) have been selected from this family on the basis of both their traditional acceptance and commercial presence (Sarin., 2003; Das and Mondal 2012). Moreover, primary screening of anti-bacterial activity has been tried with plant extracts and marker compounds. The marker compounds of these selected plants viz. [2-hydroxy 4-methoxy benzaldehyde (MBAlD)] from *H. indicus*: tylophorine and derivatives from *T. indica* and cryptolepine compounds harvested from *C. buchananii,* have other reported phototherapeutic importance (Parvatkar and Tilve., 2012; Nandy et al., 2020a; Cyriac et al., 2020). It's important to note that all these plants are listed in Medicinal Plant Conservation Area (MPCA) reports published by national and state medicinal plant boards (NMPB, Ministry of Health, Govt. of India and SMPB, West Bengal) which indicate need for strategies that would prevent their 'extinction from wild' due to overexploitation. Therefore, the aim is to establish a fast, effective and reproducible micropropagation technique, clubbed with clonal fidelity assessment, for these plants. Reportedly, the marker compounds are accumulated in mostly the root parts and then in the leaves so generation of higher amount of biomass is targeted in tissue culture system (Paulo et al., 2000; Chaudhuri et al., 2004; Prasad et al., 2017; Nandy et al., 2020b). It is reported that the specificity, concentration, treatment duration and type of elicitor molecules are capable of triggering the production of SMs in compatible growth stages of medicinal plants (Moon and Mitra, 2016). So, *in-planta* elicitation has been applied as a cost effective strategy to optimize the accumulation of valuable biomass as well as SMs. Moreover, to match the quality control parameters of the experimental process the densitometric assessment of SMs along with needful phytochemical screening is critical (Bertoli et al., 2010; Roopashree and Naik., 2019). The current work is aimed to describe high performance thin layer chromatography (HPTLC) based quantification of marker components of selected plants. Aremu et al., 2013, Ghasemzadeh et al., 2016, Xu et al., 2019, Kumar et al., 2020 has reported correlation pattern in tissue cultured plants with the activation of phenyl-propanoid pathway in association with higher SM accumulation plus enhanced bioactivity. Natural antioxidants obtained from botanicals play a crucial role in amelioration of oxidative damage caused by reactive oxygen species (ROS) (Adegbola et al., 2020). A variety of antioxidative assays and multi-faceted spectrophotometric evaluation of some major phyto-constituents viz. phenolics and flavonoids as well as antioxidant enzymes (superoxide

dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) are important in determining the antioxidative ability of plant extracts and natural compounds (Dey et al., 2020). So, the same has been followed in the present study. On the other hand, traditional reports have cited the role of medicinal plants against diabetes type diseases which has turned into "a 21st century challenge" as per Zimmet et al., 2014. The natural extracts enriched with phytochemicals are known to inhibit pancreatic lipase activity in connection to the inhibition of lipid metabolism, and obesity (Liu et al., 2020) so pancreatic lipase inhibitory assay has been standardized for the plants of interest to check whether they may substitute or complement the only clinically used pancreatic lipase enzyme inhibitor orlistat (Rajan et al., 2020).

Objectives (as specified in approved 1000 word)

1. Conservational aspect:

Establishment of fast and effective micro-propagation protocols for these important medicinal plants as an aid to their conservation along with study of regeneration and differentiation [rhizogenesis, caulogenesis, organogenesis and meri-cloning] followed by hardening, testing of clonal fidelity etc.

2. Phytochemical aspect:

Qualitative and quantitative determination of phytochemical marker compounds of selected plants. Detailed study of crude and fractioned extracts obtained from in-vivo and in-vitro grown plant and plant derived cultures.

3. Pharmacological aspect:

Estimation of relevant pharmacological properties of plant extracts derived via solvent fractionation. Pharmacological investigation on some reported ethnomedicinal properties [anti-microbial, anti-oxidant, anti-diabetic] of selected medicinal plants along with bio-controlling properties of crude plant extracts and their active component.

Methodology

1. In-vitro propagation, in-planta elicitation and Clonal fidelity assessment

1.1. Plant material, explant preparation and sterilization

The fresh and healthy plant parts (stem and leaves) were excised from the collected plants (vegetative stage; collection area: different forest, Govt. certified horticultural garden of districts of southern part West Bengal; collection time: March-September of 2015-2017). Some of collected samples were deposited in herbarium repository of Department of Life Sciences, Presidency University for future reference. The authentication was done by Dr. Avinash Mundhra and table 1A, 1B, 1C represents details of plant collection and herbarium preservation. Sterilization of all plant samples were done by applying the method described by Nandy et al., 2021.

1.2. Culture medium and growth conditions

Murashige and Skoog (MS) medium (Murashige and Skoog., 1962) fortified with sugar crystals [3% (w/v)] and plant growth regulators (PGRs) used for micropropagation of various plants. Different PGRs were added exogenously viz. 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (kin), 6-benzylaminopurine (BAP), and thiodiazuron (TDZ) (Sigma-Aldrich, Germany) 5.65 to 5.75 pH ranges were maintained. Later, 0.8%-1% (w/v) agar (Merck, India) was added for semi-solid slants and stabs used to study lateral (nodular meristemoid, callus) and vertical (rooting, elongation, multiplication, etc.) growth. For tissue culture 50 ± 5% relative humidity, 25±2° C temperature, 16h/8 h light-dark photoperiod and 60 µM/m2/s photosynthetic photon flux density (PPFD) were maintained. The experiments were repeated twice with minimum 5 explants/ trial.

1.3. Micropropagation stages: direct and indirect organogenesis

The nodal segments (2 cm in length) and leaf pieces (1.5 cm in width) were used for micropropagation in PGR fortified MS medium. The shoot induction, elongation, and multiplication were induced with different concentrations (1, 1.5, 2 mg/L) of cytokins (Kin, BAP, and TDZ). Similarly, different auxins (IAA, IBA, and NAA) were applied to induce root formation. The cytokinin and auxin rich MS medium were designated respectively as SIM (shoot induction medium) and RIM (root induction medium). The 6 weeks old leafy shoots were used as explants for rhizogenesis in RIM fortified with IAA, IBA or NAA (1, 1.5, 2 mg/L). For both auxin and cytokinin based medium, preliminary screening has showed very insignificant regeneration status under very low (0.5mg/L) and very high (2.5 mg/L) concentration of PGRs. The basal MS medium (MSO) was used as control for all types of culture, the average length plus appearance of new shoot or root outgrowths were monitored on a regular interval. Moreover, the formation of nodular meristemoids (NMs) or regenerative base callus (light green, compact) was noted and different parameters of regenerative growth like the expansion of callus diameter, accumulation of callus biomass, and shoot bud formation were documented regularly. 2,4 D (1, 1.5, and 2 mg/L) based MS-Medium was used for callus formation from leaf segments. Primary regeneration capacity of all the samples has been monitored for 8 weeks after the initiation of stage 1 of tissue culture in MSO. Later, the responsive samples were further screened for multiplication for another 6 weeks by applying cytokinin rich medium, best responsive sample got selected for next rounds of multiplication and relevant experiments. In case of indirect regeneration nodular meristemoids (NMs) has played a significant role, the differences between the fresh and dry weight (FW and DW) of NMs under the influence of different combination of auxin and cytokinin were noted.

1.4. In-planta elicitation

The plants under study were reported for their root specific secondary metabolite accumulation so the explants (healthy micro-shoots) were subjected to elicitation to check role of elicitors (if any) on accumulation of root tissue. After completion of direct and indirect organogenesis of highly responsive plant samples, *in-planta* elicitation was applied under second phase of experiment. Organic elicitors like MeJA (25, 50 and 75 µM) and SA (2.5, 5, and 10 mg/mL) were applied with RIM to induce root culture in elicitor infused root inducing medium (EI-RIM). The micropropagated plants are designated as IVPs, and the original plant sample (selected on the basis of best regenerative potential) was designated as mother plant (MP).

1.5. Acclimatization and hardening of in-vitro grown plants

All types of rooted plants, in the next phase of experiment, were acclimatized and certain field transfer protocol was followed as described by Nandy et al., 2021.

1.6. Statistical analysis

The experiments were performed thrice with minimum 3 sets (minimum 10 explants/ experiment) for direct and indirect organogenesis. The significance of difference mean values was estimated by Fisher's least (LSD) test and the Student's t-test was applied to determine the statistical significance (Pagano and Gauvreau, 2018). Values with $p < 0.05$ were considered as significant. Moreover, significance analysis of experimental model is being done with the help of multiple linear regression using XLSTAT.

1.7. DNA isolation and clonal fidelity test:

To determine the clonal fidelity, eleven IVPs (P1-P11) were randomly selected along with MP (*H. indicus*). In case of *T. indica* and *C. buchananii* the number is 7 and 9 respectively. Young leaves were used to extract DNA via modified Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987; Porebski et al 1997). 6 ISSR primers were chosen from a pool of 13 primers during initial screening based up on their ability to generate clear, scorable and reproducible bands for *H. indicus* and *T. indica*; the numbers of effective primers is 4 for *C.buchananii.* 1 kb DNA ladder was used to compare the intensity of the derived bands. The procedures of Agarose gel electrophoresis (1.0%), quality and quantity assessment of DNA and reactions for ISSR amplification were same as described by Dey et al., 2021.

1.8. Data analysis

Clear, reproducible and well resolved bands were scored with binary data matrix and the genetic similarity quotient of MPs and IVPs was calculated as per the method described by Nei and Li., 1979 to construct dendrogram using unweighted pair- group method with arithmetic mean (UPGMA) method.

2. Phytochemical diversity assessment

2.1. Plant material and extract preparation

The plant samples (MPs and IVPs) were homogenized, extracted and screened for total flavonoid content (TFC), total phenolic content (TPC), and total tannin content (TTC). The plant sample preparation process is identical as described by Nandy et al., 2021.

2.2. Total phenolic content (TPC) estimation

TPC was determined using the Folin-Ciocalteu method (Cai et al 2012) after slight modification and the spectrophotometric estimation was performed at 765 nm using gallic acid as standard.

2.3. Total flavonoid content (TFC) estimation

TFC content was determined at 415 nm using aluminium chloride ($AlCl_3$) based spectral method (Othman et al., 2014) after slight modification using quercetin as standard.

2.4. Estimation of total tannin content (TTC)

The TTC content was estimated by modified Folin–Dennis method (Bhattacharyya et al., 2014), the absorbance values were taken at 775 nm using gallotannin standard.

2.5. Determination of photosynthetic pigment content (PPC)

PPC (µg/g FW) quantification is done with the leaf tissues of MPs and IVPs as per the method of Aremu et al. 2012. Following formulae were used to estimate the content of chlorophyll a, b and carotenoids.

Chlorophyll a (C_a) = 11.24_{A662} - 2.04_{A645}
Chlorophyll b (C_b) = 20.13_{A645} - 4.19_{A662}
Total chlorophyll (C_{a+b}) = 7.05_{A662} + 18.09_{A645}
Total carotenoids = $(1000_{A470} - 1.90C_a - 63.14C_b)/214$
(C_a = concentration of chlorophyll a in µg/mL of plant extract,
C_b = concentration of chlorophyll b in µg/mL of plant extract,
C_{a+b} = concentration of total chlorophyll in µg/mL, A_z = absorbance at wavelength z)

2.6. HPTLC quantification

2.6.1 MBAlD (2 hydroxy 4 methoxy benzaldehyde)

To determine the content of aromatic vanillin type marker compound 2-hydroxy 4 methoxy benzaldehyde (MBAlD) in wild and *in-vitro* grown plants and to determine the phytochemical fidelity among the chemo-types; a validated, rapid and densitometric HPTLC quantification were performed according to the protocol described by Darekar et al., 2009 and slightly modified by Nandy et al., 2021.

2.6.1.1. Sample preparation

Freeze-dried, powdered root (1 gm), harvested from root culture (*in-vitro* grown root of *h. indicus*: IVR) and collected from MP, extracted in methanol via temperature controlled reflux process (Darekar et al., 2009; Nandy et al., 2021).

2.6.1.2. Marker stock solution preparation and experimental setup

2, 4, 6, 8 and 10 µl of methanolic stock solution (1 mg/ml) of MBAld was applied to assess the linearity which are equivalent to 200, 400, 600, 800, and 1000 ng, respectively. Pre-coated silica gel 20cm×10 cm HPTLC 60F254 plates (0.20 mm thickness) used for the calibration curve preparation with the Linomat applicator V (CAMAG, Switzerland); assisted with 100-µL application syringe (Hamilton, Switzerland) with 150 nl/s application rate; operated by WinCATS software version: 1.4.4.6337. Peak areas, plotted against corresponding concentrations and the least-squares regression analysis generated the calibration equation.

2.6.1.3. Chromatography

The root samples and the standards were loaded as 6 mm wide bands on the TLC plate with 14.4 mm average distance. 25 ml of mobile phase solution containing toluene–ethyl acetate–glacial acetic acid 7:2:1 (v/v) was to coat the TLC plates inside pre-saturated Camag twin trough chamber (10 cm × 10 cm), for 20 minutes, at 21 ºC under 50 ± 5% relative humidity and later air dried and scanned at 277 nm by CAMAG HPTLC scanner III for densitometric quantification.

2.6.1.4. Method validation

The analytical procedure was validated by linearity, accuracy, repeatability, intra-day and inter-day precision (RSD %), LOD (limits of detection) and LOQ (limits of quantification) based parameters. The R_f value, of the marker assisted in determining the specificity and absorption spectra was acquired considering the marker as 100% pure compound.

2.6.1.5. Determination of MBAld content

The densitometric quantification of MBAld in MP and IVP were done by the regression equation obtained after the HPTLC scanning and relevant procedures are same as described by Nandy et al., 2021.

2.6.1.6.Statistical analysis

All measurements were performed thrice. One-way ANOVA was done and significance of differences were calculated by using Duncan's multiple rank tests and $p < 0.05$ values were considered as significant.

2.6.2: Tylophorine

The quantification of tylophorine was performed using *in-vitro* grown root biomass (TI-IVRs) obtained from elicitor treated root culture and compared with that of MP using densitometric HPTLC procedure where sample preparation part is largely similar with that of *H. indicus* as discussed above. Marker stock solution preparation and chromatography techniques are performed with the method described by Kaur et al., 2016 (slightly modified). Toluene: ethyl acetate: ammonia (4:3.5:1.5) used as the mobile phase and HPTLC plates were air dried and scanned at 258 nm by CAMAG HPTLC scanner III for densitometric quantification.

2.6.3: Cryptolepine hydrate

The plant has not responded well in elicitor treated *in-vitro* root culture. The air dried (25–30 °C) leaf parts (1.0 g) of *C. buchananii* (IVPs and MPs) were extracted thrice with methanol and refluxed for 45 minutes in 70 °C temperature. Dried extracts were re-dissolved in 1 mL methanol and samples of varying concentration were spotted for quantification using ethyl acetate: Methanol: 35% Ammonia (8:2:1) as mobile phase and scanning was performed at 366 nm. Statistical analysis was done by using the one-way ANOVA and Duncan's multiple rank tests applied to determine the significance of differences. The measurements were taken thrice and values with $p < 0.05$, considered as significant. The other experimental set up was almost identical as *H. indicus.*

3. Pharmacological activity

3.1. Antibacterial activity

The methanolic plant extracts (MPs, IVPs) as well as methanolic solution of marker compounds were applied to assay zone of inhibition (ZOI) based anti-bacterial activity against some pathogenic bacterial strains (*Enterococcus faecalis, Escherichia coli, Shigella dysenteriae* and *Staphylococcus aureus*) following the protocol described by Mahalingam and Kannabiran., 2009.

3.2. Antioxidant activity

3.2.1. Diphenyl2-picrylhydrazyl (DPPH) radical scavenging assay

DPPH antioxidant assay recommended by Chavan et al., 2013 was performed with different plant samples. The measurements were taken at 517 nm using the following formula: % Radical Scavenging Activity = Control OD–Sample OD/Control OD X 100 (Brand-Williams et al. 1995)/ The data represented as mean ± SE (three set repeated) and carried out the analysis of variance (ANOVA).

3.2.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was conducted according to the method of Thaipong et al., 2006 after adding slight modifications and measured the absorbance of plant sample based reaction mixture at 700 nm.

3.3. Estimation of antioxidant enzyme activity

The plant samples (MPs and IVPs) were dried and powdered using liquid nitrogen and were used further for different spectrophotometric assays.

3.3.1. Superoxide dismutase (SOD) activity

The enzymatic activity of SOD (EC 1.15.1.1) was estimated by Dhindsa et al. 1981 method (modified). At first, 2 mL extraction buffer [0.5 M sodium phosphate buffer (pH 7.3), 3 M ethylene diamine tetra acetic acid (EDTA), 1.0% (w/v) polyvinyl pyrollidone (PVP), 1.0% (v/v) Tritonx100] applied on 1 g dried plant sample for homogenization which was followed by centrifugation at 10,000 rpm for 10 min at 40 ◦ C temperature. The collected supernatant was mixed with the reaction mixture [reaction buffer (1.5 mL), enzyme extract (0.1 mL), 0.2 mL methionine, 1 M NaCO3, 2.25 mM NBT solution, 3 mM EDTA, 3 mM riboflavin, and 1 mL double distilled water]. This solution was incubated at 200 ◦ C (under light from two 15 W florescent lamps) for 10 min. Later, the degree of prevention of photo-chemical reduction was measured at 644 nm. One unit of enzyme activity corresponds to 50% color disappearance and the data were expressed in enzyme unit (EU)/mg protein/h.

3.3.2. Ascorbate peroxidase (APX) activity

The modified protocol of Nakano and Asada, 1981, was applied for the estimation of APX (EC 1.11.1.11) activity. The homogenization step is similar to the previous enzymatic assay but only 0.1 g plant powder was used along with extraction solution (2 mL) and centrifuged at 10,000 rpm (40 ◦ C) for 15 minutes. The supernatant (100 µL) was added to reaction mixture [1 mL of 0.1 M sodium buffer (pH 7.2), 100 µL of 3 mM EDTA, and 0.25 mM ascorbic acid] followed by three minutes of incubation at 250◦ C. The decrease in the value of absorbance denoted the ascorbate oxidation, measured at 290 nm where the absorption coefficient is 2.8 mM/cm. The unit of enzyme activity is EU mg/protein/min which indicates the minimum amount of enzyme required to degrade 1 µM ascorbate/minute.

3.3.3. Catalase (CAT) activity

The activity of CAT (EC 1.11. 1.6) was estimated by modified method proposed by Aebl 1984. The initial steps of homogenization and centrifugation were similar and the resultant supernatant was mixed with the reaction mixture [2 mL phosphate buffer (Na-phosphate, 0.5 M, pH 7.5), 100 µL

EDTA (3 mM), 100 µL enzyme extract, and 100 µL H_2O_2]. After 3 minutes of incubation at room temperature, the absorbance was measured at 240 nm that shows CAT induced H_2O_2 decay. One unit of CAT activity is equivalent to the minimum amount of enzyme required to degrade 1 µM of H_2O_2/min where the unit of enzyme activity is EU mg/protein/min.

3.4. Pancreatic lipase inhibitory activity

The lipase inhibitory activity of MPs and IVPs was estimated via one modified method of Mc Dougal et al. 2008 (modified by De Pradhan et al., 2017). In that experiment the reaction mixture was prepared with 0.4 mL tris buffer, 150 µL lipase enzyme (exporcine pancreas), 100 µL extract as inhibitor, and 450 µL substrate (*p*-nitrophenyl laurate) which was incubated at 37 ◦ C for 2 h. Later, the solution was centrifuged at 16,000 rpm for 2 min and the supernatant was collected to measure the spectrophotometric absorbance at 400 nm. The enzyme activity related percentage inhibition was calculated using the formula "$(A_0–A_C)/A_0 \times 100$" where A_0 is absorbance without extract and A_C means absorbance with extract. A regression equation was determined from the extracts concentration to calculate the percentage inhibition plus IC_{50} value; (the concentration of sample required for 50% inhibition of the enzyme activity) using Orlistat as standard compound.

Results (Objective wise)

Objective 1. Conservational aspect:

1. *Micropropagation and direct and indirect organogenesis*

The details of plant sample (figure 1) collection from different locations of southern parts of West Bengal (figure 2) has been listed in table 1A,B,C. Initially, to generate *in-vitro* plantlets, the nodal explants and apical meristems collected from MPs were subjected to cytokinin rich MS medium or SIM and responses of shoot induction has been tabulated in table 2A, B, C. Moreover, SIM has triggered the formation of light green and compact (LG-C) NMs with better regenerative potential than the yellow and friable (Y-F) ones. *H. indicus* plants cultured in both BAP (1.5 mg/L) and KIN (1.5 mg/L) fortified SIM have exhibited significant multiplication and elongation. Number of shoots/explants was approximately 6.7 and 6.6 for BAP and KIN supplemented media respectively. Average shoot length in BAP (1.5 mg/L) fortified SIM was 11.7 ± 0.0039 cm which was the highest among all media combination (table 2A),. However, all the SIM with BAP also showed significant shoot elongation for *T.indica* (table 2B). Average shoot length was around 9cm after 6 weeks of culture. For *C. buchananii,* SIM with lowest concentration (1mg/L) of PGRs were more effective for shoot induction. Figure 3A,3B,3C, represents different micropropagation stages in *H. indicus* , figure 4A, 4B and 5A, 5B represents the same for *T.indica*, and *C. buchananii.* The correlation matrix plot (figure 6A, 6B) reflects the effect of different dosage of cytokinin on various attributes of elongation and bud break. In the second phase, after 8 weeks of culture, the influence of different doses and blends of cytokinins and auxins were studied on regeneration of shoots from nodular base callus (NBC)/ NMs also which has been noted in case of *H.indicus* and *T.indica* (table 3A, 3B). Presence of IBA in media along with different cytokinin triggered the formtaion of NMs and promoted the emergence of shoot initials. 2-4-D (1 or 2 mg/L) fortified media promoted callus generation from excised leaf segments in all three plants (figure 3B, 4A, 5B). figure 7A,7B present the 'Box-Whisker' plot showing the distribution pattern of changes in fresh weight (FW) of NMs under different

concentration and combination of cytokinins and auxins in case of *H.indicus* and *T. indica* respectively. The role of IBA as promoter for NM formation and effective for expansion of NM biomass with both BAP and KIN is evident as per the graph but the role of BAP is also significant as depicted by the 3rd and 4th box (from Y axis) of figure 7A and 7B. Highest biomass gain was observed in combination of BAP (2 mg/L) and IBA (0.50 mg/L). KIN-IBA combination induced biomass gain beyond 1g in larger number of samples; however, for BAP-IBA combination the distribution is almost equal. The expansion in callus diameter and increase in fresh weight (especially for green compact NMs) positively impacted emergence of shoot initials also as depicted in Table 3A and 3B in case of both *H. indicus* and *T. indica*. Thus, BAP fortified media demonstrated the highest biomass gain; however, biomass gain in IBA free media was not very different compared with the IBA-fortified media; only higher concentration of IAA showed some noteworthy data (Table 3A, 3B). The formation of NMs, are not prominent in case of *C. buchananii* probably due to its slow growth pattern in both natural and *in-vitro* system.

2. Effect of in-planta elicitation

According to the table 4A, 4B and 4C, one can detect the most potent auxin for root induction in these medicinal plants. After primary screening with all collected plant samples it has been noted that IBA and IAA can induce rooting response in these medicinal plants, and later it was observed that IBA is way more potent than IAA. Highest root length in 1.5mg/l IBA was found to be 8.2±0.011 cm, 9.3±0.015 cm and 3.2±0033 cm respectively for *H. indicus, T. indica* and *C. buchananii.* During *in-planta* elicitation, the results of rooting response of *H. indicus* obtained from IBA fortified medium was compared with post-elicitation effects. It has been observed that in case of *H.indicus* and *T. indica* MeJA based *in-planta* elicitation has significantly improved root length but the lowest concentration (25 µM) did not generated any significant impact on rhizogenesis. On the other hand, no significant root inducing effect has been observed for SA based elicitation for *H.indicus.* Figure 8A and 8B is the scatter plot of root length versus root number in *H. indicus* and *T. indica* which depicts the effect of elicitation on rooting. The plot (figure 8A) clearly demonstrates that 50 µM MeJA (Set 1) can increase the root length but results obtained after applying 75 µM MeJA (Set 2) was not better than the effect of IBA infused RIM in case of *H. indicus*. On the other hand if we look into the results of MeJA elicitation for *T.indica* we would see IBA infused RIM and both Set 1 and 2 has exhibited almost the same result; but IBA significantly increased the total number of roots (figure 8B). In case of *T. indica* also IBA is the prime root inducing PGR as per table 4B. However, in case of *C. buchananii,* rooting response was very slow in RIM (table 4C) and no significant result have been found in case of MeJA or SA elicitation.

3. Experimental model and its significance analysis

The entire tissue culture experiments with all these medicinal plants are dependent on a number of developmental patterns of direct and indirect organogenesis, which may have some significant effect on the outcome. To determine the effect of various attributes the multiple linear regression has been performed where root length has been considered as a dependent variable and 'number of shoot/explant', 'length of shoot', 'number of root/explant' and 'base callus percentage' have been considered as explanatory variables. As per the statistical analysis, coefficient of determination is

0.897, which is definitely a good fit to understand the relationship between the variables. The analysis of standardized coefficient has established the influence of different attributes on overall root length of *H.indicus* (figure 9A) As per the Fisher test of the analysis of variance the equation of the experimental model is root length = 7.807+0.470*no of shoot/explant-1.447*length of shoot-0.270*no of root/explant+7.055E-02*base callus percentage. The predictability of the experimental model has been determined for *T.indica* also (figure 9B) where total number of root and base callus formation has positively influenced the root length and equation has been determined. The statistical analysis of goodness of fit of the experimental model applied on *C.buchananii* has showed the R^2 (coefficient of determination) is only 0.773 where shoot length and number of root per explant has significantly influenced the outcome (figure 9C).

4. Clonal fidelity assessment

In *H. indicus, T. indica* and *C. buchananii,* ISSR markers to assess the clonal fidelity of *in vitro* raised plants and donor mother plant table 5 A, B and C reveals the details of tested primers while figure 10 A, B and C describes the ISSR banding pattern. Out of the 13 ISSR primers only 6 primers produced 12 clear and reproducible bands with an average of 2.8 bands per primer and band size ranging from 300-2600bp. UBC-112 produced the highest number of 6 bands per primer (table 5A). 3 ISSR primers (UBC-117,UBC-126, UBC 130) showed similar banding profiles between donor mother plant and in vitro progenies revealing 100% monomorphic characters; for *T. indicus* UBC 103 and UBC 130 were most effective in showing monomorphism. For *C. buchananii* only 4 primers were effective, 2 of them has shown less than 20% degree of polymorphism. Figure 10 D, E and F shows the closeness of randomly selected plant samples with their respective mother plant based on Nei and Lei's coefficient of similarity derived from ISSR marker analysis represented with UPGMA.

Objective 2. Phytochemical aspect:

1. Phytochemical diversity assessment

The variability in phytochemical content of collected plant material along with EI-IVPs and IVPs is summarized in table 6 A,B,C. The variations in TPC, TFC, and TTC have clearly demonstrated that the phytochemical profile of micropropagated plants are more or less similar with mother plants. For both *H. indicus* and *T. indica* the significance of elicitation is also evident on phytochemical levels. In case of *C. buchananii* the values of TPC, TFC, and TTC did not showed any significant changes when compared with MPs. HI-KOL, showed significant TPC, TFC, and TTC values. Moreover, in all cases the changes of TFC values were marginal after elicitation. Figure 11 represents the calibration curves and respective regression equation utilized to estimate TPC, TFC, and TTC content in wild and regenerated samples of *H. indicus, T. indica* and *C. buchananii*.

2. Photosynthetic pigment content profiling

The effect of elicitation on chlorophyll and carotenoid content is not at all significant as per the table 7 but it is important to observe that *in-vitro* condition is capable to produce enough

photosynthetic pigment which is at par with the naturally grown plant. Only the micropropagated *C. Buchananii* plants has showed lesser concentration of total chlorophyll and carotenoid pigments.

3. Densitometric quantification of marker compounds via HPTLC

A simple, robust, and reproducible densitometric HPTLC quantification for the marker compounds has been described here. Table 8A, 8B present the method validation parameters, intra-day and inter-day precision screening, and recovery studies to quantify the content (mg/g) of MBAld accurately from methanolic root extracts of MP or HI-KOL and HI-IVR. The quantitative estimation primarily has proved the positive role of elicitation and micropropagation on secondary metabolite accumulation. Figure 12A represents the chromatograms developed for MBAld quantification by using methanolic root extracts of MP (3.30±0.1 mg/g) and IVPs (3.32±0.1 mg/g) at 277 nm. The resolution of tylophorine with clear and reproducible peak recorded at R_f 0.68 (table 8C, 8D). The methanolic root extract of MP and TI-IVR produced calibration curves, at 258 nm linear in the range of 200, 400, 600, 800, 1000, 1,200 ng for tylophorine. In wild and *in-vitro* sample the content of tylophorine is almost identical 3.53±0.5 mg/g and 3.52±0.3 mg/g respectively (figure 12 B). To assay cryptolepine hydrate leaf tissues were used. Detailed method validation parameters and chromatograms are presented in table 8E and 8F and figure 12C. The content of marker compound was comparatively higher in wild *C. buchananii* leaf (2.19±0.1 mg/g) than the micropropagated ones.

Objective 3. Pharmacological aspect:

1. Antibacterial activity

The results obtained from agar well diffusion assay with crude plant extracts was not reproducible or noteworthy but standard compounds has shown significant growth inhibition against tested pathogenic strains of microorganisms. Table 9 represents average ZOI obtained by applying three different concentrations of the marker compounds. MBAld and cryptolepine found to be more active against *Staphylococcus aureus* and *Shigella dysenteriae* and showed a concentration dependent growth inhibition.

2. Antioxidant activity

The DPPH and FRAP based antioxidant assays have shown promising result in *in-vitro* counterparts of all selected medicinal plants where the observed data (table 10) is quite at par with the wild plants. For *H. indicus* and *C. buchananii*, wild plants have showed slightly better result while in case of *T. indica*, the results are almost the same.

3. Enzymatic activities

In this study the data of antioxidant enzyme activity (SOD, CAT, and APX) is quite at par with anti-oxidant assays and total content of active phytochemicals. In figure 14 (B, C, D) the graphs represents the enzyme activity pattern observed in MPs and IVPs of selected medicinal plants. To determine the catalase activity standard curve of H_2O_2 has been used (figure 14 A) In all cases, *H. indicus* IVP sample has showed better activity than the wild ones which may be induced by *in-vitro*

culture condition but in case of *C. buchananii* reversed result has been observed. For *T. indica* the enzymatic activities has remained overall similar between naturally grown and *in-vitro* grown counterparts.

4. Assessment of lipase inhibitory activity

In the present investigation, the effect of naturally and *in-vitro* grown plant samples on lipase inhibitory property have been screened. To determine the role of plant extracts as natural lipase inhibitor, the IC50 value of MP, and IVPs were determined and compared with standard, orlistat. Moreover, the data for same activity of the commonly known and commercially popular lipase inhibitor, *Gymnemma sylvestre* extract has also been determined and compared. It was clearly observed that IC50 values of plant samples were much higher than the standard but *H. indicus* showed comparatively significant lipase inhibitory activity in a dose dependent manner (table 11, figure 15). The highest activity (lowest IC_{50} value) was observed in HI-IVP which was slightly higher than that of the extract (100 µL) prepared from the *H. indicus* MP. The lipase inhibitory activity present in other plant samples which is quite lower than the standard.

Discussion

The selected medicinal plants valued in Indian pharmacopeia for their ethno-medicinal acceptance among the wide tribal population of West Bengal and different parts of India (Manna and Ghosh., 2021). In a large section of Indian subcontinent, the ethnic tribes use crude plant extract, root powder paste or decoction and mixture of these plants against a number of ailments (Nandy et al., 2020a). They have their presence in costemetic, pharma- and nutraceutical based food industries which has reduced their presence in wild due to large scale exploitation (Van Andel and Havinga., 2008). In the present study, a rapid and reproducible micropropagation protocol has been developed as an ex-situ conservational tool for these rapidly declining medicinal plants which are mostly cultivated for alkaloid rich root parts. Moreover, the reproducibility of the experimental model has been ascertained by several statistical analysis which have identified the co-dependence of different variables associated with growth of the root parts. In addition, *in-planta* elicitation, phytochemical diversity assessment, and demonstration of biological activities of various plant samples were also carried out. Reportedly, the biological activities ascribed to these plants are an attribute of their phytochemical content (Soetan and Aiyelaagbe., 2009). For commercial plants which are harvested for particular plant part or particular type of secondary metabolite, elicitation could trigger the production of specific SMs (Narayani and Srivastava., 2017). In case of *H. indicus*, the root part is most important owing to the higher content of methoxybenzaldehydes (Nandy et al., 2020a, b) and same is noted for *T. indica* and *C. buchananii* (kaul et al., 2003; Gantait and Kundu., 2017) with their respective marker tylophorine and cryptolepine. Phenolics and flavonoids are reported for their free radical scavenging properties with a significant physiological role in plants (Huang et al., 2009). Presence of diverse phytochemicals or SMs in the plant extracts or herbal formulations function as effective antioxidant implicated to the efficacy of traditional formulations (Koc et al., 2015). It is known that the induction of oxidative damage has proved to be linked with a number of chronic ailments i.e. cancer, rheumatism, neuro-degeneration, diabetes, renal failure, and cardiac arrests (Erel et al., 2014). In the present study, plants demonstrating higher TPC or TFC

values also exhibited promising results in DPPH and FRAP assays. The present *in-planta* elicitation for production of more biomass and higher TPC and TFC content in micropropagated plantlets can be implicated to a cross talk that stimulates 'value addition' process of these medicinal plants. *T. indica,* mostly known as anti-asthmatic plants, has been studied under *in-vitro* condition but detailed analysis of antioxidant enzyme activities or role of IBA in huge mass of nodular meristemoids generation, especially for this plant has been reported for the first time. *C. buchananii* is comparatively less known plant, a rich source of bioactive indoquinoline type of chemicals (cryptolepine, isocryptolepine, neocryptolepine) (Chaves et al., 2017). ISSR marker based polymorphism assessments, screening of TPC, TFC, TTC as well as analysis of antioxidant enzyme activities is represented in the current work. Weissner, 2014 and Nandy et al., 2020a reported the consumption of root based decoction or aqueous root sherbet (Nannari syrup) containing *H. indicus* as health boosting drink. Moon et al., 2020, reported phenolic compounds present in Khus drink or aqueous extract of *Vetiveria zizanioides* L. Nash could potentiate the nutritive value of the same. One particular step, the temperature controlled reflux, has been followed for both spectrophotometric assays and HPTLC quantification of MBAlD, tylophorine or cryprolepine. The heat treatment reportedly releases phenolics and consequently influences the antioxidant enzymatic activity (Seok-Moon et al., 2004). Heat-mediated release of free or linked esters from cell wall and the cleavage of glycosidic bonds present in different types of plant material may be attributed to the enhanced TPC content in the experimental plant samples (Xu et al., 2007). In DPPH free radical scavenging assay, superior activity of few extracts was detected even visually by observing gradient wise formation of light blue color. However, the FRAP assay or 'redox-linked colorimetric reaction' involves determining the ability of extracts or natural products to reduce Fe3+ ions to Fe2+. In both DPPH and FRAP assays, the data obtained from the *in-vitro* grown plants are largely compatible with their naturally grown counterparts. Almost similar observation was noted by studying the activity of antioxidant enzymes and pancreatic lipase inhibition. The plants and their marker compounds are already reported for anti-diabetic activity (Tirumani et al., 2018). However, no report is available on enzymatic inhibition of pancreatic lipase. The root powder and the decoction prepared from the medicinal plant are reportedly consumed for their multi-faceted health-promoting properties. Recent studies have shown polyphenol rich medicinal plants may act as natural lipase inhibitors (Seyedan et al., 2015). The actual molecular role played by MeJA is not clear from the present study but it was evident that elicitation could potentiate the regeneration capacity or rhizogenesis response of the regenerated plant extracts. In Milk thistle MeJA based elicitor feeding could enhance marker and phenolic compound accumulation in tissue culture (Gabr et al., 2016). Emergence of multi-drug resistant microorganisms has triggered the search for novel antibiotics and natural products from the Apocynaceae plant family demonstrating antioxidation mediated antimicrobial activities (Anand et al., 2020) and according to the study at least MBAld may play a major role in this regard. Development of photosynthetic pigments in the leaf tissues of micropropagated plant is a valuable marker since the plants are cultured under artificial light and controlled environment where light intensity and photoperiod are externally regulated (Nandy et al., 2021). Another important drawback is the size of the leaves which is directly influenced and many times shunted by space-limitation inside culture vessels, lack of optimum microelement in culture medium, overcrowded culture (especially in multiplication experiments) etc. which reduce the density of photosynthetic pigments in cultured leaf tissues and may play a vital role in plant

survival after hardening. Since there is not much difference in pigment content between MP and IVP, it indicates that during the hardening and acclimatization periods, in-vitro raised plants develop their photosynthetic machinery in a robust way to ensure their survival. Moreover, precise elicitor treatment could potentiate the use of these medicinal plants as important component of combination therapy or synergism against drug. Although multi-directional use of these medicinal plants were reported, but no multi-faceted assessment has been conducted for the plants growing in southern region of Bengal. The densitometric HPTLC validation of the marker content could determine the % content which could easily be used as a parameter for detection of adulterants and effective quality control for large scale harvesting of these plants. The densitometric quantification procedure described here is simple, precise, robust, accurate, and validated. Therefore, the evaluation of marker content using recommended desitometric HPTLC based quantification can further be used for identification of phytochemical fidelity, selection of elite chemotypes that ensure the quality of germplasms which are more profitable for commercial harvesting.